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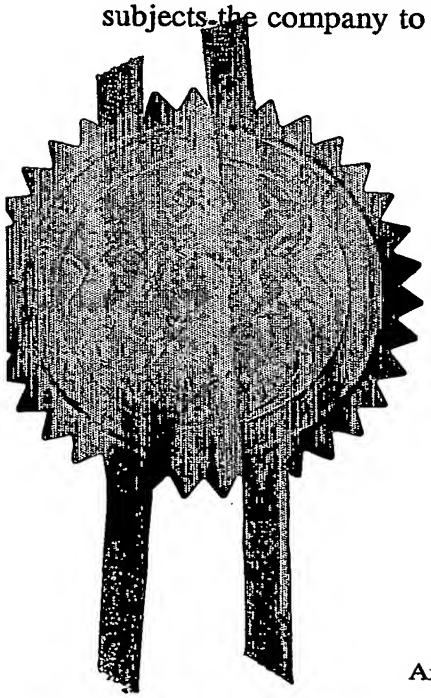

Signed P. Mahoney

Dated 27 May 2003

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The Patent Office
Cardiff Road
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NP10 8QQ

1. Your reference — MJL/C1277/C

2. Parent application number *(The Patent Office will fill in this part)* — 04 SEP 2002 — 0220452.7

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Cytocell Limited
Unit 6 Somerville Court
Trinity Way
Adderbury
Banbury Oxon OX17 3SN 06048755003

Patents ADP number *(if you know it)* (See Continuation Sheet)

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom 06715221001

4. Title of the invention — Lateral Flow Assay Device and Method (II)

5. Name of your agent *(if you have one)* — Keith W Nash & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

90-92 Regent Street
Cambridge CB2 1DP

Patents ADP number *(if you know it)* — 1206001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
GB	0216162.8	12/07/2002

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*
-	

8. Is a statement of inventorship and of right to grant of a parent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

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Continuation sheets of this form	
Description	22
Claim(s)	4
Abstract	1
Drawing(s)	3

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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	Continuation Sheet

11. I/We request the grant of a patent on the basis of this application.

Signature Keith W. Nash & Co Date 04/09/2002

Keith W Nash & Co

12. Name and daytime telephone number of person to contact in the United Kingdom: M J Lipscombe (01223) 355477

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Continuation Sheet

Continuation of Box 3: Further Applicants

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Golden Gate, Tyglas Avenue,
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United Kingdom 06715221001

DUPLICATE

C1277.00/C

Title: Lateral Flow Assay Device and Method (II)

Field of the Invention

The present invention relates to a lateral flow assay device that may be used to detect the presence and/or amount of a target nucleic acid sequence in a sample, a kit comprising the lateral flow device, and a method of performing an assay.

Background of the Invention

Sensitive detection of nucleic acids has advanced over recent years with the development of a variety of nucleic acid detection and amplification techniques. These amplification techniques can be broadly divided into specific target amplification and signal amplification. Examples of target amplification techniques include, Polymerase Chain Reaction (PCR) (US 4,683,195 and US 4,683,202), Nucleic Acid Sequence Based Amplification (NASBA) (US 5,130,238), and Transcription Mediated Amplification (TMA) (US 5,399,491). Examples of signal amplification techniques include, Signal Mediated Amplification of RNA Technology (SMART) (WO 93/06240), Split Promoter Amplification Reaction (SPAR) (WO 99/37805), Invader (US 5,846,717) and Ligase Chain Reaction (LCR) (EP 0,320,308). These technologies can be further subdivided by their ability to amplify target / signal either by requirement for thermal cycling (such as PCR) or ability to operate at a single temperature (isothermally).

One commonality between these techniques is the requirement for front end sample preparation (nucleic acid extraction) and back end amplicon detection. All of these techniques involve multi-step processes to achieve detection of the amplicon, and current development is targeted at complex systems for automation of these processes, to provide medium to high throughput of analytes.

Nucleic acid amplification techniques have been well described and illustrated in the prior art and all rely on the action of nucleic acid dependent enzymes. One such technique, SMART (WO 93/06240), relies on the interaction of two probes combined with the sequence of interest to form a three way junction (TWJ) structure generating an RNA signal after the action of DNA polymerase and an RNA polymerase. The RNA signal generated from the TWJ may be further amplified by linear amplification probes, (see WO 01/09376). Detection of the RNA signal may be achieved by a number of means that are well described in the prior art that include, but is not limited to, molecular beacons (US 5,925,517), latex beads, FRET /DFRET.

For some of the aforementioned processes, expensive, complex equipment is required together with a level of skilled labour to perform such techniques. For these techniques to become widely used for both clinical and industrial applications, reduction in the complexity of the tests (i.e. number of steps and skill base required) together with a reduction in the instrumentation and cost per test are required.

Specifically, for these tests to be applicable at the near patient (point of care-PoC), or near process level, simple, easy to use, cost competitive systems are required.

Chromatographic or lateral flow assays have been used for many years to simplify the performance of tests such that they can be performed by semi- or unskilled users and require minimal equipment; they are therefore ideally suited to PoC tests. To date, however, their application has primarily been restricted to immunoassays that are less complex than nucleic acid tests since they are simply detection assays, there being no amplification step.

Lateral flow tests typically utilise a single, capillary device that contains some (or preferably all) of the reagents necessary for the performance of an assay. These reagents are typically contained within discrete zones of the device, such that as fluid flows along the device by capillary flow the various reactions occur sequentially and a signal is

generated at a detection zone that is indicative of the presence and/or amount of analyte in the sample. The devices can be a single membrane with reagents deposited at specific sites (e.g. US 4,161,146; US 4,361,537), or be composed of a series of discrete pads or membranes (each containing none, one or more reactants) arranged such that their edges are in liquid contact with one another (EP 0186799).

A typical lateral flow device will contain a sample receiving zone (which may contain buffers and chemicals necessary for the test), a label zone (which contains an analyte-binding reagent, such as an antibody, releasably bound to the membrane), a capture and detection zone (which contains an analyte-binding reagent immovably immobilized on the membrane), and an absorption zone or sink of sufficient capacity to enable unbound labelled reagent to wash out of the detection zone. The pads or membranes are typically attached to an impervious backing, and the pads or membranes are in liquid contact with one another (usually achieved by overlapping the edges and use of adhesive or a lamination layer). Optionally the device is encased in a protective housing with defined apertures for sample application and visualization of result. Examples of such devices include those disclosed in US 5,656,503; US 5,622,871; US 5,602,040; US 4,861,711.

To perform such an assay, sample is added to the sample receiving zone where it is drawn into the device by capillary force. Filter devices incorporated into the sample application zone can be used to remove blood cells, etc., and act as a volume control device (EP 0186799). The sample then hydrates and mixes with a labelled binding reagent (e.g. chromophore-labelled antibody), and any analyte present in the sample reacts with this in a specific manner to form a labelled analyte complex. This complex migrates along the device to the detection zone where a second binding reagent, immobilized on the strip, binds to the labelled analyte complex and prevents further migration of the labelled analyte complex. Unbound labelled binding reagent is drawn through the detection zone to the absorption zone. Thus, presence of signal at the detection zone is indicative of presence of analyte in the sample.

A variety of labels have been used for lateral flow assays, including radioactive (US 4,361,537) and fluorescent labels (US 6,238,931), although visual labels are preferred for PoC applications. These include enzyme-generated colourimetric signals (US 4,740,468) and particulate chromophores (US 5,591,645; US 4,943,522; US 5,714389).

Nucleic acids based analysis utilising lateral flow techniques has been described in the prior art. For example, WO 00/12675 discloses an assay system that integrates nucleic acid extraction, specific target amplification and detection. The device described therein comprises a hollow elongated cylinder with a single closed end and a plurality of chambers therein. Nucleic acid extraction and amplification steps occur within the cylinder. The amplified product is then contacted with the proximal end of a lateral flow test stick in order to perform the detection step of the assay. Accordingly, the device disclosed in WO 00/12675 is relatively complex.

Patent application No. US 2001/0036634 discloses an apparatus for performing a nucleic acid assay which assay involves a thermocycling amplification reaction (e.g. PCR). The apparatus comprises a lateral flow test stick and an associated thermally-regulatable apparatus such that as a nucleic acid amplification reaction mixture migrates along the test stick it passes through a plurality of stationary thermal zones, such that the reaction mixture is thermally cycled in a manner suitable to perform a polymerase chain reaction. The nucleic acid amplification reaction mixture is prepared outwith the assay apparatus and is applied to a sample receiving portion of the lateral flow test stick. Thus, the test stick disclosed in US 2001/0036634 does not include an integral nucleic acid extraction zone.

Summary of the Invention

In a first aspect the invention provides a lateral flow assay device to test for the presence and/or amount of a nucleic acid sequence of interest in a sample, the lateral flow device comprising:

(a) a sample receiving zone for contacting the device with a sample to be tested;

(b) a nucleic acid amplification zone in liquid communication with the sample receiving zone; and

(c) a detection zone for detecting the product/s, directly or indirectly, of a nucleic acid amplification reaction performed in the amplification zone, said detection zone being, or being locatable, in liquid communication with the amplification zone, wherein an extraction step is performed on or within the lateral flow assay device.

In a second aspect the invention provides a lateral flow assay device to test for the presence and/or amount of a nucleic acid sequence of interest in a sample, the lateral flow device comprising:

(a) a sample receiving zone for contacting the device with a sample to be tested;

(b) a nucleic acid isothermal amplification zone in liquid communication with the sample receiving zone; and

(c) a detection zone for detecting the product/s, directly or indirectly, of an isothermal nucleic acid amplification reaction performed in the amplification zone, said detection zone being or being locatable, in liquid communication with the amplification zone.

The assay device in accordance with the second aspect defined above differs from the assay device of the first aspect in two regards:

(i) the amplification reaction performed using the device of the first aspect may be (and preferably is) isothermal but may alternatively involve thermal cycling, whilst the amplification reaction performed using the device of the second aspect is solely isothermal;

(ii) an extraction step is performed on or within the sample receiving zone of the device in accordance with the first aspect – such a step may optionally also be performed (and preferably is performed) on or within the sample receiving zone of a device in accordance with the second aspect. Alternatively, however, an extraction step may be performed separately from the assay device and the resulting extracted sample subsequently applied to the sample receiving zone.

In a third aspect the invention provides a method of detecting the presence and/or amount of a nucleic acid sequence of interest in a sample, the method comprising the steps of:

contacting a sample comprising the sequence of interest with the sample receiving zone of an assay device in accordance with the first aspect of the invention so as to cause a nucleic acid amplification reaction to take place in the nucleic acid amplification zone of the device; and detecting, directly or indirectly, the product/s of the amplification reaction in the detection zone of the device.

In a fourth aspect the invention provides a method of detecting the presence and/or amount of a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting a sample comprising the sequence of interest with the sample receiving zone of an assay device in accordance with the second aspect of the invention so as to cause a nucleic acid amplification reaction to take place in the amplification zone of the device; and detecting, directly or indirectly, the product/s of the amplification reaction in the detection zone of the assay device.

In a fifth aspect, the invention provides a method of making a lateral flow assay device in accordance with the first and/or second aspects of the invention defined above, the method comprising the steps of: forming a porous matrix comprising an amplification zone and a detection zone, said amplification zone being, or being locatable, in liquid flow communication with a sample receiving zone, the sample receiving zone comprising one or more reagents immobilised or releasably bound thereon so as to perform a nucleic acid extraction step on a nucleic-acid containing sample contacted with the sample receiving zone. Typically the one or more reagents comprise one or more (preferably all) of the following: a detergent; a base: a chelating agent; and a free radical trap. These reagents are described in greater detail elsewhere.

In a sixth aspect the invention provides an assay kit for performing an assay to test for the presence and/or amount of a nucleic acid of interest in a sample, the kit comprising a lateral flow assay device in accordance with the first and/or second aspect of the invention, and a supply of at least one reagent required to perform the assay. Conveniently the kit may additionally comprise a supply of carrier liquid, which is applied to the device during performance of the assay. The said at least one reagent may be supplied ready dissolved

or suspended in the carrier liquid, or may be supplied, for example dried or lyophilised, preferably in ready-to-use aliquots. The reagent(s) supplied with the kit, separate from the assay device may be, for example, one or more of the following: a DNA polymerase; an RNA polymerase; an analyte-specific nucleic acid probe; an amplicon-specific labelling reagent; rNTPs; dNTPs and the like.

The assay device of the invention and associated aspects will now be further described. Unless the context dictates otherwise, the description below will generally apply equally to an assay device in accordance with either the first or the second aspects of the invention.

The "extraction" step may comprise one of more of the following:

(i) lysis of bacterial, plant, animal, human, yeast or other fungal cells present in the sample;

(ii) disruption of any viral particles present in the sample;

(iii) at least partial purification of the nucleic acid in the sample (e.g. comprising separation of nucleic acid from fragments of lipid membrane and/or removal of polypeptide contaminants); and

(iv) inactivation of DNase and RNase present in the sample.

The amount and nature of extraction required will depend at least in part on the nature of the sample and the nature of the target sequence of interest. For example, where the analyte is double stranded a thermal (or more preferably) chemical denaturation step, typically as part of the extraction, is required to yield single stranded nucleic acid amenable to assay. Agents suitable to cause such a chemical denaturation may conveniently be present (e.g. immobilised or releasably bound) to the sample receiving zone. Alternatively, where the target is single stranded (typically, single stranded RNA) then no such thermal or chemical denaturation step will normally be required.

Once the target has been rendered single stranded, it will normally be advantageous for the target to be treated in some way to reduce the likelihood of reassociation of the strands. This could involve, for example, dilution in carrier liquid to reduce the concentration of

the target strands and/or rapid contact with a considerable excess of one or more target specific probes.

In a device in accordance with the first aspect of the invention, the nucleic acid extraction is performed on or in the lateral flow device. This is also a preferred feature of a device in accordance with the second aspect of the invention. This arrangement has the advantage that essentially all the steps of the assay may be performed on the lateral flow assay device, providing greater simplicity than prior art arrangements.

The extraction step preferably occurs in and/or near the sample receiving zone. In particular, it is preferred that the sample receiving zone comprises agents which are capable of achieving the desired extraction. Typically such agents are releasably bound or immobilized on and/or within a porous matrix. Such agents desirably include one or more (preferably all) of the following: a detergent, a base, a chelating agent, and a free radical trap. Details of suitable agents are contained within US Patent Nos. 5,496,562; 5,807,527; 5,985,327; 5,756,126; and 5,972,386. Typically the detergent is an anionic detergent, conveniently SDS (sodium dodecyl sulphate). The base is advantageously a weak base, typically monovalent. Note that the base may be provided as its corresponding salt (preferably a carbonate) and the term 'base' as used herein should be construed accordingly where the context so permits. A preferred base is Tris (i.e. tris-hydroxymethyl methane). A preferred chelating agent is EDTA (i.e. ethylene diamine tetra-acetic acid). The free radical trap is less significant than the detergent, base and chelating agent. A suitable free radical trap is uric acid or a urate salt. It may be particularly useful in situations where the sample is not processed immediately after contacting with the assay device but is left for some time (e.g. to be archived) before any amplification reaction takes place. In other situations the free radical trap may normally be avoided.

In a preferred embodiment, the lateral flow assay device in accordance with the first or second aspects of the invention, comprises an FTA matrix or similar, preferably in the sample receiving zone. FTA paper is available from Whatman International Limited

(Maidstone, Kent, UK) and comprises a cellulose-based matrix coated with agents (such as those described above) which lyse cell and nuclear membranes, denature polypeptides and inactivate enzymes (such as nucleases) and which protect nucleic acid from UV-mediated or other environmental damage. FTA paper has been described in detail in the US patents referred to in the preceding paragraph. A similar material, known as IsoCode(RTM), is available from Scheicher & Schuell.

For the purposes of illustration, a suitable sample receiving/extraction matrix comprises a cellulose-based paper, such as filter paper, having a minimal loading (per square cm of paper) as follows: SDS 1mg; Tris 8 micromols (968.8mg of free base); EDTA 0.5 micromols (146.1mg free acid) and, optionally, uric acid 2 micromols (336.24mg).

Nucleic acids present in the sample become temporarily entrapped within the matrix. Wash or carrier liquid (e.g. TE buffer) can be added to wash away the unrequired contaminants. Care must be taken that the amount of fluid added is not so great as to saturate the lateral flow assay matrix and wick (normally present), otherwise subsequent capillary flow will not be possible.

The FTA paper or similar such material may be restricted to the sample receiving zone or may constitute all or a substantial part of the porous matrix of the lateral flow assay device. The amplification reaction may take place on the FTA paper – the reagents which inactivate enzymes etc. being washed off the matrix by the application of wash or carrier liquid. Alternatively, nucleic acid temporarily entrapped within the FTA matrix may be eluted (by application of for example, Tris-EDTA buffer or other aqueous EDTA-containing solution to the matrix) and thence transported into a downstream portion of the lateral flow device comprising a generally conventional nitrocellulose or similar porous matrix, within which the amplification reaction may be performed. Alternatively, in a device in accordance with the second aspect of the invention, the sample receiving zone may be an essentially inert conventional matrix (e.g. nylon or nitrocellulose) to which a pre-extracted nucleic acid-containing sample is applied.

The nucleic acid sequence of interest may comprise DNA, RNA, or mixtures thereof and may be a naturally occurring molecule or a synthetic molecule. Typically the sequence of interest may be derived from an infectious disease agent of man or animals, food spoilage organisms, or from animal (especially mammalian), human or plant sources. The assay devices find particular application as diagnostic tools to assist in diagnosis of infectious diseases or other pathological conditions (e.g. diagnosis of genetic disorders or conditions associated with particular genetic abnormalities) and in the detection of spoilage organisms in foods or detection of pathogens or markers of faecal contamination (e.g. *E. coli*) in water or other environmental samples. Thus the sample applied to the sample receiving zone of the assay device may be, or be derived from (as appropriate), any sample of interest. The sample may typically be a biological sample (e.g. blood, plasma, serum, urine, sweat or the like), or a sample of food or drink, or an environmental sample such as a water sample or a swab from a surface.

The lateral flow assay device of the invention is typically a low-cost item and disposed of after a single use. Generally the assay device comprises a permeable or porous matrix which at a proximal end is in liquid communication with the sample receiving zone, such that liquid applied to the sample receiving zone may flow along the device through the permeable or porous matrix by virtue of capillary action. It is conventional to provide a highly absorbent "sink" or wicking member at the distal end of the porous matrix, to enhance the capillary flow. Analytes and/or reagents suspended or dissolved in the liquid may be transported along the device by the flow of liquid.

Typically the porous matrix and wicking member are substantially enclosed within an impervious casing, often comprising an opaque plastics material, to facilitate handling of the device and to protect the matrix against contamination.

Again, it is conventional to provide lateral flow assay devices with at least one test reagent which is releasably bound in and/or on the porous matrix, typically in dessicated or lyophilised form, such that contacting the assay device with a liquid will release the test reagent which may then be transported by the capillary flow of the liquid along the porous

matrix. It is also conventional to provide at least one test reagent, typically a capture reagent, in the detection zone of the device, which reagent is immobilised in and/or on the porous matrix, such the flow of liquid along the matrix will not release the reagent in question. This facilitates concentration and detection of an analyte in the detection zone.

The assay devices of the present invention will normally possess these conventional features. The general principle of operation of the devices of the invention is that a nucleic acid sequence of interest present in a sample applied to the sample receiving zone will be transported, by capillary flow along the porous matrix to the nucleic acid amplification zone where, typically dependent on the presence of the sequence of interest, a nucleic acid amplification reaction will take place. The amplified product/s of that reaction (known as the amplicon/s) will typically become labelled in an amplicon-specific manner, and will continue to pass along the porous matrix to the detection zone, where the amplicon/s will be captured by an immobilised capture molecule.

Those skilled in the art will appreciate that it is necessary for liquid to be present in order to perform the assay. The sample may itself be in liquid form. Alternatively it may be necessary to add a carrier liquid to the sample either prior to contacting the sample with the assay device, or *in situ* on the sample receiving zone. The carrier liquid will normally be aqueous and may include, for example, distilled or deionised water, or an aqueous buffer solution, such as TE buffer. The carrier liquid may be added all in one go, or be added in discrete aliquots (this latter option may usefully be employed to help control the flow of the analyte and/or reagents along the assay device). Conveniently the carrier liquid may comprise one or more of the reagents required to perform the assay (e.g. RNA or DNA polymerases; rNTPs or dNTPs; probes; labels etc). In addition, or alternatively, as explained above, carrier liquid may be applied to the device to wash away contaminants from the sample receiving zone and/or to elute away from the sample receiving zone agents useful for performing the extraction step but which may inhibit the subsequent nucleic acid amplification reaction. The amount of carrier liquid applied to the device will typically be in the range 50μl-2ml, preferably in the range 100μl-1ml.

Generally, the arrangement will be such that liquid (together with associated analytes and/or resuspended reagents) will flow from one zone to another, allowing various steps of the assay to be performed sequentially. This flow may be essentially continuous at a substantially constant speed. However it may be preferred to cause a discontinuous flow, with different flow rates at different points along the porous matrix, e.g. to allow certain reaction products to accumulate before they proceed to the next zone of the device. Variation of the flow rate may be achieved by any of a number of suitable means, including but not limited to, a physical switch, a dissolvable barrier, restriction of capillary flow (e.g. by altering the porosity/permeability of the matrix) and the like. Examples of fluid control systems used in immunoassay lateral flow assays, and which may be employed in the present invention, include the use of chemical gates (US 6,271,040), centrifugal force (US 4,989,832), capillary restrictions (US 6,271,040), separate fluid channels of differing pathlengths for reagents (US 4,960,691; US 5,198,193), or physical means (e.g. the WheatRite test from C-Qentec). The porous matrix may be provided as a single continuous strip of may be formed from two or more portions which are held, or locatable, in liquid communication so as to provide a liquid flow path from one portion to an adjacent portion.

In one embodiment the lateral flow assay device comprises means to alter the relative positions of two or more portions of the porous matrix, so as to affect the rate of flow of liquid from one portion to another. This may comprise, for example, a plunger or push-button which can be actuated to bring previously separated portions of the matrix into liquid flow communication with one another.

The porous matrix may comprise, for example, cellulose and/or cellulose derivatives (especially nitrocellulose), although any suitable porous material (e.g. nylon, polysulfone) may be used. Preferably the porous matrix is provided with a backing material (typically a piece of plastics sheet material, such as Mylar) to provide increased strength and rigidity. Typically the porous matrix may be treated with conventional agents to prevent non-specific binding/absorption of analyte or reagents. Suitable blockers of non-specific binding include polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP).

In some embodiments the lateral flow assay device will comprise at least one reagent which is required for the nucleic acid amplification reaction, which reagent is provided releasably bound to the porous matrix in, or upstream of, the amplification zone. In some embodiments at least one reagent, required for the nucleic acid amplification reaction, is provided suspended or dissolved in a carrier liquid which is applied (typically at the sample receiving zone) to the lateral flow assay device. Moreover, it is also possible that at least one reagent required for the amplification reaction may be immobilised on or in the porous matrix (i.e. such that flow of a liquid along the matrix will not release the reagent). For example, oligonucleotide or polynucleotide probes, primers and the like may be immobilised to an amino-activated matrix by phenyldiisothiocyanate (PITC) or disuccinimidyl suberate.

It will be noted that the embodiments described immediately above are not mutually exclusive and may be combined in any combination e.g. wherein one or more reagents requires for the nucleic acid amplification reaction may be releasably bound to the matrix, one or more may be immobilised on or in the matrix, whilst one or more other reagents may be present in a liquid applied to the lateral flow assay device. Typically the amplification reaction will require: the target sequence of interest; at least one nucleic acid probe which comprises a portion complementary to the target sequence; at least one nucleic acid polymerase; and nucleotide triphosphates which may be utilised by the nucleic acid polymerase to synthesise a polynucleotide or oligonucleotide.

The nucleic acid amplification zone is that part of the assay device in which all the essential components of the amplification reaction are brought together so that, in suitable conditions, the amplification reaction occurs. Thus, the amplification zone may or may not be a clearly discernible or discreet portion of the lateral flow assay device. In particular, the amplification zone may be co-extensive with, or form part of, the sample receiving zone.

Further the amplification reaction may be one which results in amplification (i.e. synthesis of multiple copies) of the target sequence or one which results in amplification of a signal sequence, generation of the signal sequence being ultimately dependent on the presence of the target sequence of interest in the sample. Examples of target sequence amplification techniques which may be employed include PCR, NASBA (US 5,130,238) and TMA (US 5,399,491). Examples of signal sequence amplification techniques which may be employed include SMART (WO93/06240) SPAR (WO99/37805) and Invader/Cleavase (US 5,846,717).

In a device in accordance with the second aspect of the invention it is an essential feature that the amplification reaction is an isothermal reaction (i.e. one performed at a substantially constant temperature, without thermal cycling). In a device in accordance with the first aspect of the invention it is a preferred feature that the amplification reaction is an isothermal reaction. The isothermal amplification reaction may take place at room temperature (e.g. 20°C) or may take place at some other temperature. In order to increase the speed of the reaction and/or increase the stringency of hybridisation it may be preferred to perform the reaction at an elevated temperature (e.g. at a temperature in the range 30-50°C). Since thermal cycling is not required a simple 'hot block', oven, water bath or other incubator may be used to heat the assay device and hold it at the desired temperature for the requisite period of time.

A preferred isothermal amplification technique is a signal amplification method. In particular, preferred amplification reactions comprise SMART (as disclosed in WO 93/06240) and/or SPAR (as disclosed in WO 99/37805). Both these techniques require the use of at least one nucleic acid probe which comprises a sequence which is complementary to the sequence of the target of interest.

In the case of SMART, two such probes are employed, each being complementary to a different, but adjacent portion of the target nucleic acid, such that in the presence of the target the two probes (one a "template" probe, the other an "extension" probe) become hybridised adjacent to each other on the target, in a complex known as a "three way

junction". The hybridisation of the two probes in close proximity allows the further hybridisation of respective 'arm' portions of the probes to each other. One of these arms (the arm of the "template" probe) is longer than the other (the shorter arm being that of the "extension" probe). This allows the shorter of the two arms to be extended, using the larger arm as a template, by a DNA-dependent DNA polymerase in the presence of dNTPs. Extension of the arm creates a double stranded portion of nucleic acid which comprises an RNA polymerase promoter sequence (e.g. one recognized by T7, T3 or SP6 RNA polymerases).

Thus, in the presence of a suitable RNA polymerase and rNTPs, multiple RNA copies of one of the probes are formed. This results in "signal" amplification, and the multiple RNA copies may themselves be further amplified, if desired, by any one of a number of amplification processes known to those skilled in the art (e.g. as disclosed in WO 01/09376). The RNA copies, or amplified copies thereof, may then typically be captured and detected in the detection zone.

Accordingly, both dNTPs, rNTPs, DNA polymerase RNA polymerase and suitable buffers may be required, as well as template and extension probes. Conveniently, the majority of these reagents will be provided in a carrier liquid applied to the sample receiving zone of the assay device and/or releasably bound to the porous matrix of the device. In one embodiment one of the probes (preferably the template probe) is immobilised in or on the porous matrix and the other reagents are present in a carrier fluid applied to the sample receiving zone and/or releasably bound to the porous matrix.

Labelling

Conveniently the amplicon/s (i.e. the amplified end product/s of the amplification reaction) becomes associated with a readily detectable label upstream of the detection zone. The label may be any suitable substance that is readily detectable e.g. a radio label or an enzyme label. It is however greatly preferred that the label is a direct visible label (i.e. one which is apparent to an observer without any prior processing) such as particulate

coloured "latex" (in actuality, these "latex" particles are polystyrene) or colloidal gold particles.

It is obviously desirable that the labelling is amplicon-specific. One of the simplest ways of achieving this is to ensure that the amplicon has a sequence which is essentially unique amongst the nucleic acids entering a labelling zone and to provide a labelling reagent which comprises a base sequence complementary to that of the amplicon, such that the labelling reagent hybridises to the amplicon in a sequence-specific manner.

Desirably the labelling reagent is provided releasably bound to the porous matrix, upstream of the detection zone, such that as amplicon migrates along the assay device it becomes associated with the labelling reagent which is released by the capillary flow of liquid, the complex of amplicon and labelling reagent then migrating to the detection zone.

The labelling moiety will conveniently comprise, in addition to the label, a moiety which is a member of a specific binding pair ("sbp"). Such sbps are well known to those skilled in the art and include antigens/antibodies, complementary strands of nucleic acid, ligands/receptors and the like. A preferred sbp for present purposes is biotin/strepavidin.

Detection

The labelled amplicon is detected in the detection zone. This is conveniently achieved by immobilising on the porous matrix a capture molecule which is specific for the labelled amplicon complex (more particularly, specific for the amplicon). The amplicon-specific capture molecule may be any molecule which can bind in a specific manner to the amplicon and which may be immobilised on the porous matrix. Conveniently the amplicon-specific capture molecule may comprise a nucleic acid sequence complementary to that of the amplicon or may comprise a nucleic acid binding protein (e.g. a "zinc finger" polypeptide) or a sequence-specific anti-DNA or anti-RNA antibody (or effective binding portion thereof, such as an Fab, Fv, scFv etc.).

The amplicon-specific capture molecule will conveniently be immobilised in a line or band across the porous matrix, substantially tranversely arranged relative to the direction of liquid flow along the assay device. Accordingly, labelled amplicon will be captured and concentrated, forming a visible line in the detection zone. It is perfectly possible, however, to deposit the capture molecule in other configurations, so as to form, for example, a spot or other shape. In addition, it is possible to arrange the device so as to deposit a capture molecule in two or more locations. If desired, two or more different capture molecules may be deposited at respective locations, each capture molecule being specific for a respective amplicon, such that a single device can be used to test for the presence and/or amount of a respective number of different sequences of interest. Naturally, the amplification reagents to amplify the different targets (or sequences derived therefrom) will need to be provided also.

It is also preferred that the assay device comprises a control zone, advantageously downstream of the detection zone. The control zone typically comprises an immobilised capture reagent which binds specifically to a reagent which participates in the amplification and/or detection reactions or which might be generated by a control nucleic acid amplification reaction. One convenient arrangement is for the control zone to comprise a line or band of immobilised reagent which exhibits specific binding for the labelling reagent (e.g. the labelling reagent may be a biotinylated oligonucleotide and the immobilised control zone capture molecule comprises streptavidin).

In an alternative embodiment the capture zone comprises an immobilised array of capture molecules which capture excess labelled amplicon.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, in which:

Figures 1, 2 and 3 are schematic representations of various embodiments of an assay device in accordance with the first and/or second aspects of the invention.

Detailed Description of Embodiments

Example 1

Referring to Figure 1, an assay device in accordance with both the first and second aspects of the invention comprises a lateral-flow assay strip, indicated generally by reference numeral 1. The strip is provided with a backing of clear synthetic plastics material, such as Mylar sheet.

The strip is substantially enclosed within a casing of opaque plastics material, forming a protective casing 2 (denoted by the broken line). The casing has an aperture 4 at a proximal, upstream end of the device and a window 6 towards the distal, downstream end. The aperture 4 allows the lateral flow strip to project beyond the casing, at which proximal end there is a sample receiving zone 8. The window 6 allows a user to observe the formation of a test result signal at the test line 10 and a control result signal at the control line 12. The sample receiving zone 8 comprises Whatman FTA paper, which material is useful for performing an extraction step, so that the sample receiving zone 8 is in effect a combined sample receiving and extraction zone.

The sample receiving zone 8 is in liquid flow communication or contact with a porous matrix denoted generally by reference numeral 14, which is itself in liquid flow contact with a wicking member 16 of highly absorbent material (e.g. Ahlstrom 222 in a pad of dimensions approximately 5mm by 20mm).

Adjacent to, and slightly overlapping with, the combined sample receiving and extraction zone 8 is an amplification zone 18 which comprises a pad of Whatman GF/C porous material comprising reagents for performing an isothermal SMART nucleic acid amplification, the reagents comprising:

(i) Template probe attached to 2μm latex microparticles (amine-modified and crosslinked via phenyldiisothiocyanate);

(ii) Extension probe; (iii) DNA polymerase; (iv) RNA polymerase; (v) dNTPs; (vi) rNTPs; (vii) Linear amplification probe; and (viii) amplicon-specific labelling probe coupled to 40nm gold colloid, prepared by incubation of 40nm gold colloid (British BioCell) with thiol-capped probe for 1 hour, following by blocking excess binding sites with 1mg/ml BSA.

A mixture containing (i)-(viii) at the appropriate concentrations is prepared in transcription buffer comprising 160mM Tris (pH 7.8), 24mM $MgCl_2$, 8mM spermidine, 40mM DTT, 600mM NaCl, 0.002% Micrococcus DNA, 1% Ficoll and 1% PVP, also containing 5%w/v sucrose, and 50μl dispensed onto the pad. The pad is then dried by lyophilisation.

The amplification zone 18 is adjacent to, and slightly overlapping with, detection zone 20. The overlap ensures good liquid flow communication between the respective zones of the porous matrix 14. The detection zone 20 comprises a strip of nitrocellulose (HF 135 Millipore) 5mm x 25mm. Immobilized on the nitrocellulose at test line 10 is an amplicon-specific capture molecule, in the form of a probe oligonucleotide complementary to the sequence of the amplicon. The test line 10 is formed by suspending the amplicon-specific capture probe in 25mM phosphate buffer (pH 7.0) containing 0.5mg/ml BSA, and depositing a stripe of the suspension across the nitrocellulose, which is then dried overnight at 21°C at a relative humidity of less than 20%.

The control line 12 may be formed in a substantially similar manner, using a capture molecule specific for the labelling probe.

The combined sample receiving and extraction zone 8, amplification zone 18, and detection zone 20 are laminated onto adhesive-backed Mylar sheet (from Adhesives Research) to provide support and ensure their correct orientation. Liquid flow between the components is ensured by providing a 2mm overlap between adjacent components. The components 8, 18 and 20, with their Mylar backing are placed within a moulded synthetics plastics material which forms protective casing 2. Internal projections within the casing 2

at the points of overlap ensure good liquid flow communication between adjacent components.

A large number of variants of the illustrated embodiment can be readily envisaged e.g. the use of a moiety, such as a nucleic acid probe (especially a SMART assay template probe) bound to labelled latex particles which are deposited in dry form on the porous matrix of the assay device and which are mobilised on contact with a carrier liquid and hence migrate along the assay device whereupon they may be captured by a capture moiety deposited on a control line which has specific binding activity for a moiety on the template probe or the latex particle on which it is supported, thereby forming a visible control result signal at the control line, providing a visible indication to the test user that sufficient liquid has been contacted with the sample receiving zone to mobilise the reagent(s) releasably bound to the porous matrix.

Example 2

Assay for *E. coli* 23S rRNA

This example relates to an assay device and method in accordance with the invention, for the detection of *E. coli* 23S rRNA. The apparatus is illustrated schematically in Figure 2, in longitudinal section. Components of the illustrated apparatus analogous to the embodiment represented in Figure 1 are denoted by the same reference numerals.

As before, a combined sample receiving and extraction zone 8 (comprising FTA paper), an amplification zone 18, a detection zone 20 and a wicking member 16 are laminated onto a piece of adhesive-backed Mylar 22 and substantially enclosed within a moulded plastics coating (not shown).

At the junction of the amplification zone 18 and the detection zone 20, a 2mm gap is left between the portions which are adhered to the Mylar backing 20. A non-adhered flap 24 of the amplification zone 18 is provided, 5mm in length. The flap 24 overlaps the

detection zone 20 but liquid flow communication between the amplification zone 18 and the detection zone 20 is initially prevented by the presence of an intervening removable sheet 26 of impermeable plastics material, which at least partially projects through an aperture provided in the casing. The aperture may be the same as result window 6 (in Figure 1) or be a separate aperture.

To perform an assay, sample is added onto the sample receiving and extraction zone 8 and the device placed on a heated block at 41°C for lysis and release of nucleic acids. Carrier fluid (e.g. TE buffer) is then added from a dropper bottle or micro pipette, causing the extracted nucleic acid to migrate by capillary action to the amplification zone 18 and mobilise the reagents releasably bound therein. Typically between 50μl to 2ml of carrier fluid is added, preferably between 100μl and 1ml.

Following the mobilisation of the amplification reagents within the amplification zone 18 by the carrier fluid containing the released nucleic acid, generation of amplicon ensues if the target (23S rRNA from *E. coli*) sequence is present. As the amplicon is generated by the amplification reaction, it binds to a colloidal gold-labelled amplicon-specific probe to form a labelled amplicon/amplicon-specific probe complex.

Because the liquid flow communication with the detection zone 20 is blocked by the removable plastics sheet 26, the amplicon generated, and the resulting labelled complex, accumulate in the amplification zone 18.

After 40mins, the plastics separation sheet 26 is removed, allowing liquid, together with labelled amplicon and any excess free labelled amplicon detection probe to migrate into the detection zone 20. Conveniently, a projection and/or biasing member is provided on the inner surface of the casing, to urge the amplification zone 18 into intimate contact with the detection zone 20 once the impermeable plastics sheet 26 is removed. Any labelled amplicon present becomes bound to the amplicon detection probe immobilised at the test line 10 and forms a red-coloured line. The free labelled amplicon detection probe migrates

past the test line and is captured by a probe-specific capture moiety immobilised at the control line 12, forming a visible control result.

The test and control lines can be visualized through a window in the casing (or quantified by a reader), and a red line is indicative of presence of *E. coli* 23S rRNA target in the sample.

Example 3

A further embodiment is illustrated schematically in Figure 3. Again, components of the assay device which are analogous to those shown in Figure 2 are denoted by common reference numerals.

In this embodiment there is provided an air gap between the amplification zone 18 and the detection zone 20. After an appropriate amount of time has elapsed, to allow amplicon to accumulate in the amplification zone 18, the relative positions of at least part of the amplification zone 18 and the detection zone 20 are altered to establish liquid flow communication therebetween. In the particular embodiment illustrated in Figure 3, the positions of at least a flap part 24 of the amplification zone 18 is altered, relative to the detection zone 20, by actuation of a plunger or push-button 28 which is received within an aperture 30 provided in the casing 2. Depression of the plunger or push-button 28 causes the component to bear down on the flap 24, pushing it into intimate contact with the detection zone 20, thereby allowing liquid and any accumulated amplicon and other mobilised substances to pass into the detection zone 20.

It should be noted that in the embodiments illustrated in Figures 2 and 3, interruption of the liquid flow path between the amplification zone 18 and the detection zone 20 also has the result of removing the wicking effect of wicking member 16. It is important therefore that the amplification zone 18 is of reasonable absorbency to provide sufficient capillary flow to draw analyte and/or reagents from the sample receiving/extraction zone 8.

Claims

1. A lateral flow assay device to test for the presence and/or amount of a nucleic acid sequence of interest in a sample, the lateral flow device comprising:

(a) a sample receiving zone for contacting the device with a sample to be tested;

(b) a nucleic acid amplification zone in liquid communication with the sample receiving zone; and

(c) a detection zone for detecting the product/s, directly or indirectly, of a nucleic acid amplification reaction performed in the nucleic acid amplification zone, said detection zone being, or being locatable, in liquid communication with the amplification zone; characterised in that a nucleic acid extraction step is performed on or within the lateral flow assay device.

2. An assay device according to claim 1, wherein the nucleic acid amplification comprises an isothermal amplification reaction.

3. A lateral flow assay device to test for the presence and/or amount of a nucleic acid sequence of interest in a sample, the lateral flow device comprising:

(a) a sample receiving zone for contacting the device with a sample to be tested;

(b) a nucleic acid isothermal amplification zone in liquid communication with the sample receiving zone; and

(c) a detection zone for detecting the product/s, directly or indirectly, of an isothermal nucleic acid amplification reaction performed in the amplification zone, said detection zone being, or being locatable, in liquid communication with the amplification zone.

4. A lateral flow device according to claim 3, further characterised in that a nucleic acid extraction step is performed on or within the lateral flow assay device.

5. A lateral flow assay device in accordance with claim 1 and further in accordance with claim 3.

6. A lateral flow assay device in accordance with any one of the preceding claims, wherein the device comprises one or more reagents releasably bound on a porous matrix.

7. A lateral flow assay device according to claim 6, wherein the one or more reagents releasably bound comprise one or more reagents required to perform the nucleic acid amplification reaction.

8. A lateral flow assay device according to any of the preceding claims comprising one or more reagents immobilised on a porous matrix.

9. A lateral flow assay device according to claim 8, wherein the one or more immobilised reagents comprise an amplification-specific capture moiety.

10. A lateral flow assay device according to any one of the preceding claims comprising a releasably bound or an immobilised probe comprising nucleic acid.

11. A lateral flow assay device according to any one of the preceding claims, wherein the sample receiving zone comprises reagents suitable to perform a nucleic acid extraction step on a sample applied to the sample receiving zone.

12. A lateral flow assay device according to any one of the preceding claims comprising FTA paper or equivalent material.

13. A lateral flow assay device according to any one of the preceding claims, comprising means to cause interruption of flow, or alteration of rate of flow, of a liquid along a porous matrix within the device.

14. A lateral flow assay device according to claim 13, comprising means to alter the relative positions of two or more portions of the porous matrix, so as to affect the rate of flow of liquid from one portion to another.

15. An assay kit for performing an assay to test for the presence and/or amount of a nucleic acid sequence of interest in a sample, the kit comprising a lateral flow assay device in accordance with any one of the preceding claims and a supply of at least one reagent required to perform the assay.

16. An assay kit according to claim 15, comprising a supply of carrier liquid.

17. An assay kit according to claim 16, wherein said at least one reagent is provided dissolved and/or suspended in the carrier liquid.

18. A method of performing an assay to test for the presence and/or amount of a nucleic acid sequence of interest in a sample, the method comprising the steps of: contacting the sample with the sample receiving zone of a lateral flow assay device in accordance with any one of claims 1-14, so as to cause a nucleic acid amplification reaction in the presence of the sequence of interest; and detecting, directly or indirectly, the product/s of the amplification reaction in the detection zone of the lateral flow assay device.

19. A method of making a lateral flow assay device in accordance with the first and/or second aspects of the invention defined above, the method comprising the steps of: forming a porous matrix comprising an amplification zone and a detection zone, said amplification zone being, or being locatable, in liquid flow communication with a sample receiving zone, the sample receiving zone comprising one or more reagents immobilised or releasably bound thereon so as to perform a nucleic acid extraction step on a nucleic-acid containing sample contacted with the sample receiving zone.

20. An assay device substantially as hereinbefore described and with reference to the accompanying drawings.

21. An assay kit substantially as hereinbefore described and with reference to the accompanying drawings.

22. An assay method substantially as hereinbefore described and with reference to the accompanying drawings.

C1277.00/C

Abstract

Title: Lateral Flow Assay Device and Method (II)

Disclosed is a lateral flow assay device to test for the presence and/or amount of a nucleic acid sequence of interest in a sample, the lateral flow device comprising:

(a) a sample receiving zone for contacting the device with a sample to be tested;

(b) a nucleic acid amplification zone in liquid communication with the sample receiving zone; and

(c) a detection zone for detecting the product/s, directly or indirectly, of a nucleic acid amplification reaction performed in the nucleic acid amplification zone, said detection zone being, or being locatable, in liquid communication with the amplification zone; characterised in that a nucleic acid extraction step is performed on or within the lateral flow assay device.

Fig. 1

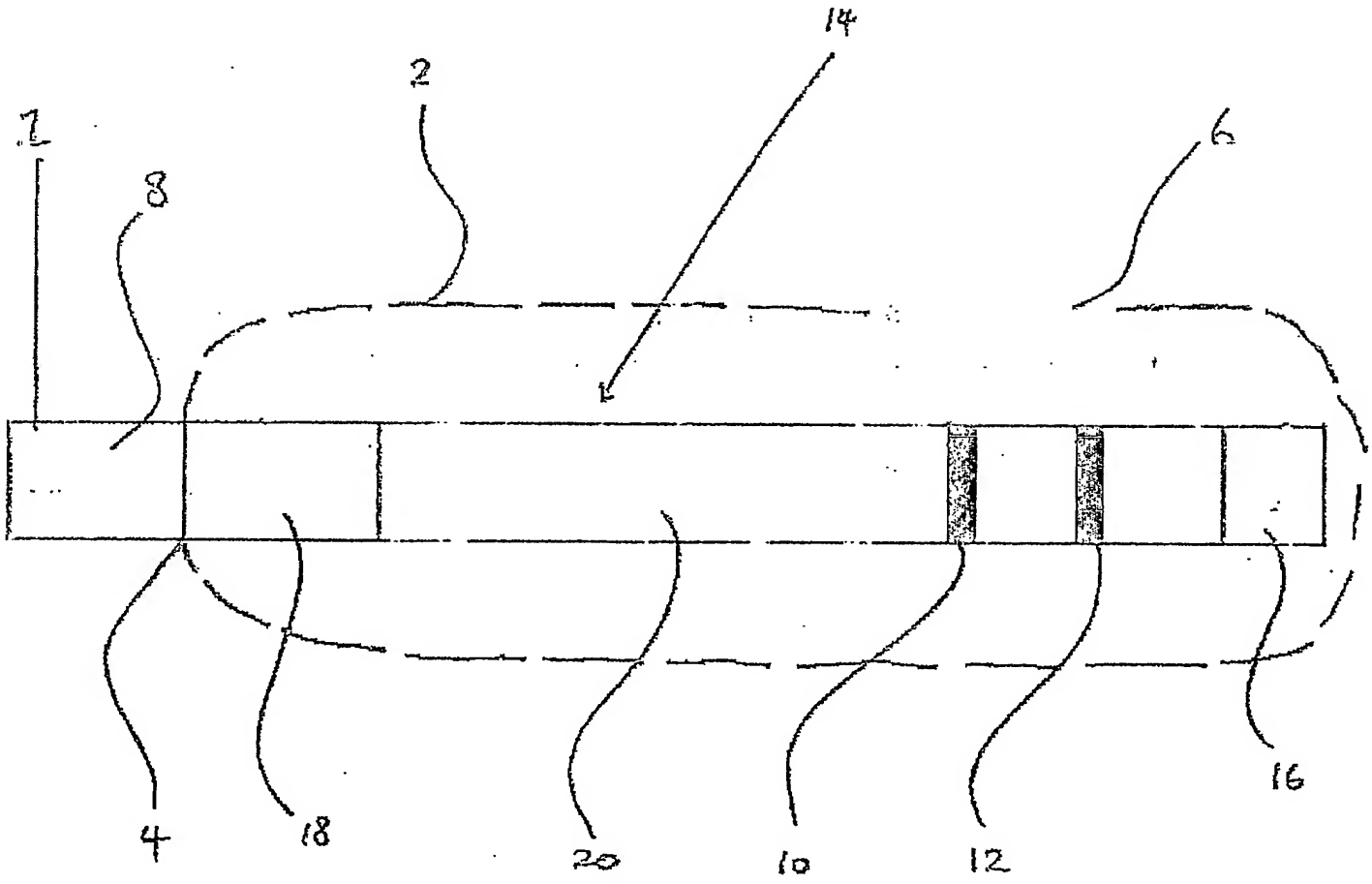


Fig. 2

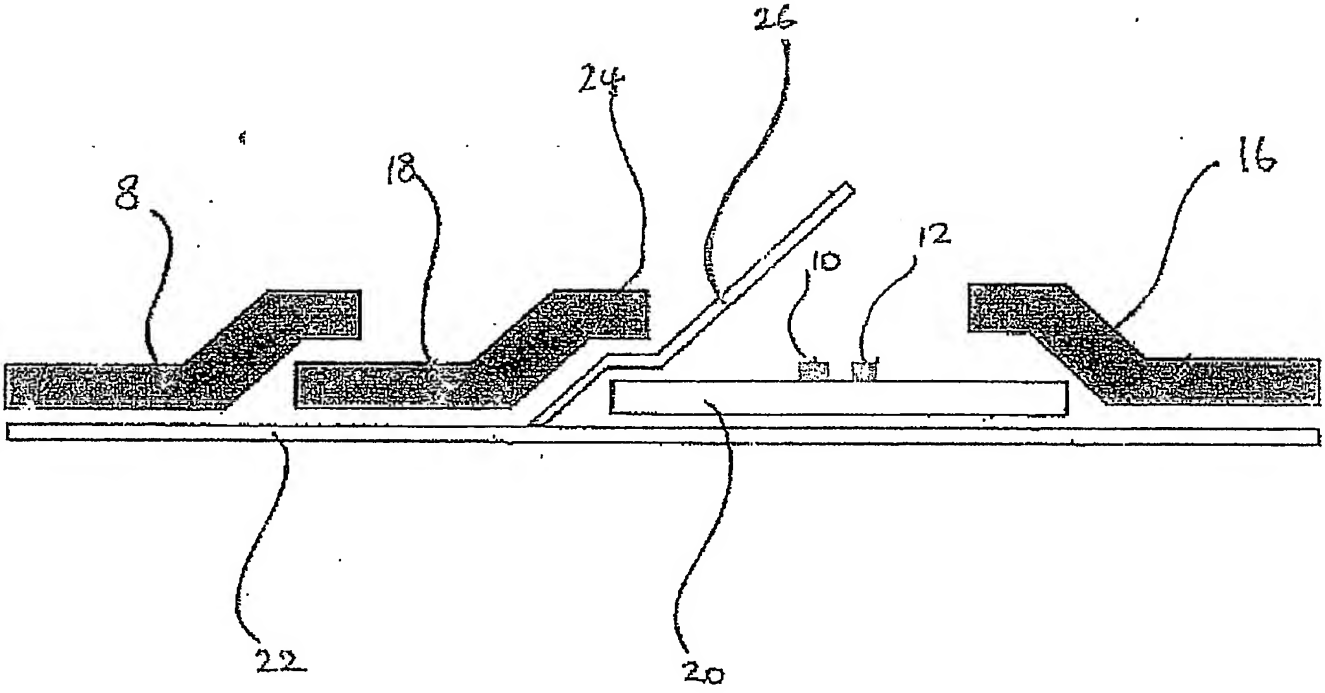
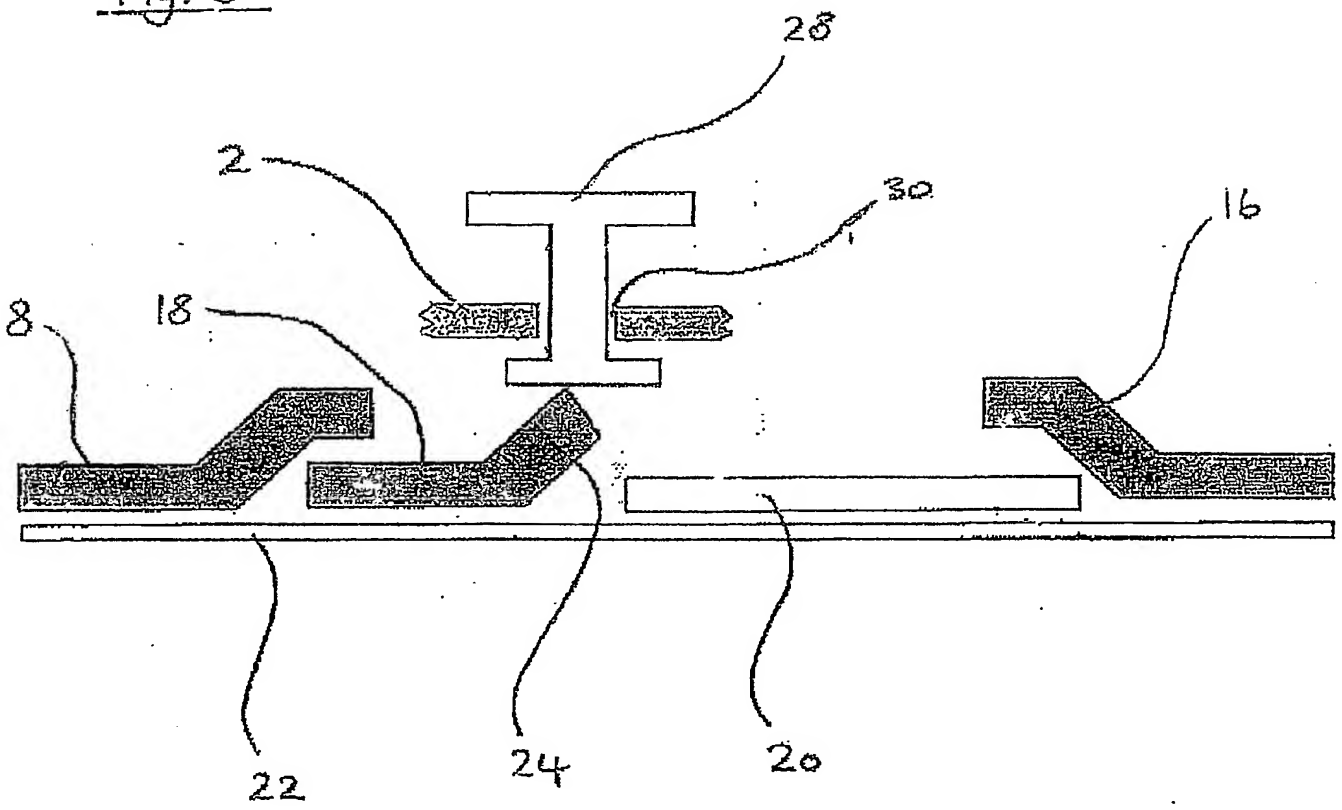


Fig. 3



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